IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Elena Babiychuk, et al.	) Group Art Unit:	1638
	)	
Application Number: 10/705,197	) Examiner:	COLLINS, C.E
	)	
Filed: November 12, 2003	) Confirmation No.:	3003
	)	
For: Methods and Means To Modulate Programmed Cell Death in Eukaryotic cells	)	

DECLARATION UNDER 37 C.F.R. SECTION 1.132

Second Supplemental Declaration by Dr. Marc DE BLOCK

I, Marc De Block, hereby declare that:

1. My credentials are set forth in the Supplemental Declaration provided during the prosecution of the parent application USSN 09/118,276.

2. I have been informed that in the US Official Action dated May 19, 2006, the Examiner has rejected the pending claims because these claims contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains to make and/or use the invention.

3. It is my understanding that the rejections in the Official Action are based in part on the doubts by the Examiner that:
   a. the downregulation of *parp* genes by dsRNA encoding genes to obtain plant with high vigor can be obtained in plant cells using dsRNA encoding chimeric genes which are directed to part of the *parp* genes different from those exemplified in the patent application;
   b. the downregulation of *parp* genes by dsRNA encoding genes to obtain plant with high vigor can be obtained in plant cells using dsRNA

encoding chimeric genes targeted to parts of the *parp* genes smaller than 300 consecutive nucleotides; and

c. the downregulation of *parp* genes by dsRNA encoding genes to obtain plant with high vigor can be achieved in plant cells other than oilseed rape and Arabidopsis.

4. Attached as Annex I, is a paper I have co-authored entitled "Poly(ADP-ribose) polymerase in plants affects energy homeostatis, cell death and stress tolerance" which was published in The Plant Journal, 2005, Volume 41, pages 95-106.

5. The above mentioned paper describes data from experiments, performed according to the teaching of the application, which I have also provided in my Supplemental Declaration provided during the prosecution of the parent application 09/118,276.

6. For ease of reference, I would like to point out the following correspondence in terms used in the patent application, my supplemental declaration and the publication:

   a. A *parp* gene of the ZAP class, as used in the application, is referred to as a *parp1* gene in the publication, in accordance with international conventions;

   b. A *parp* gene of the NAP class or specifically with regard to Arabidopsis an *app* gene, as used in the application, is referred to as a *parp2* gene in the publication, in accordance with international conventions;

   c. The h*pATParp1* construct described in the publication in detail on page 103, 1st column in the section entitled "Experimental procedures – Plasmid constructions corresponds to the to the chimeric gene indicated in my Supplemental Declaration paragraph 15.iii as pTYG48.

   d. The h*pATParp2* construct described in the publication in detail on page 103, 1st column in the section entitled "Experimental procedures – Plasmid constructions corresponds to the to the chimeric gene

indicated in my Supplemental Declaration paragraph 15.i as pTYG29. The nucleotide sequence of *parp2* from *Arabidopsis* is the nucleotide sequence set forth in SEQ ID No 5 of the patent application.

7. h*pATparp1* and h*pATparp2* encode a dsRNA region targeted towards the N-terminal region of respectively the *parp1* and *parp2* coding regions (nucleotides 428-955 of *parp1* and nucleotides 189-781 of *parp2*).

8. h*pATparp2(signature)* encodes a dsRNA region targeted towards the PARP signature in the C-terminal region of the *parp2* coding region of Arabidopsis (corresponding to SEQ ID No. 5) (nucleotides 1572-1730 of *Atparp2*).

9. Transgenic *Arabidopsis* plants comprising the h*pATparp2(signature)* chimeric gene exhibit in up to 40% of the transgenic lines a high tolerance to high light stress i.e. these plants are more vigorous (see publication page 97, left column, lines 26 to 28).

10. These data demonstrate that indeed different parts of the PARP gene may be targeted for down regulation through dsRNA mediated gene silencing and can be successfully used for obtaining highly vigorous plants.

11. These data also demonstrate that a part of the PARP gene which is smaller than 300 bp (*in casu* about 158 bp) may be targeted for down regulation through dsRNA mediated gene silencing and can be successfully used for obtaining highly vigorous plants.

12. Attached as Annex II are selected slides from a slide deck which I used for a presentation at the Plant Biology and Biotechnology Symposium entitled "Recognition and Signal Transduction" San Diego Center for Molecular Agriculture, October 24&25, 2005. The title of my presentation was "Improving stress tolerance and yield – A genetic and an epigenetic approach".

13. The first slide I have selected is a schematic representation of PARP proteins and an indication of the part of the encoding DNA which is targeted by the different dsRNA encoding chimeric genes. I refer to paragraph 6 for an explanation of *hpAtparp1, hpAtparp2, hpAtparp2signature. hpZmparp1* refers to the chimeric gene described in my Supplemental Declaration paragraph 15.ii as pTYG33. pTYG33 comprises a part of the nucleotide sequence of SEQ ID No 1 of the patent application. *hpZmparp2* refers to a chimeric gene encoding a dsRNA corresponding to part of the sequence of SEQ ID No. 3 of the patent application.

14. The second slide summarizes data on drought stress tolerance in the field obtained for transgenic corn lines comprising the *hpZmParp1* and *hpZmparp2* chimeric genes described in paragraph 13. The bars represents the yield of control and transgenic corn lines under drought conditions expressed as a percentage of the yield obtained for these lines in irrigated conditions. Corn lines comprising the chimeric genes *hpZmParp1* and *hpZmparp2* provide less loss of yield under drought conditions than non-transgenic control lines.

15. These data demonstrate that the teachings of the application can be applied to obtain highly vigorous transgenic corn plants which provide a higher yield under low-irrigation conditions in the field.

I also declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States and that such willful statements may jeopardize the validity of this application or any patent issued thereon.

16 November 2006

Date

Marc De Block

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Poly(ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance

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Summary

Plants contain two genes that code for poly(ADP-ribose) polymerase (PARP): *parp1* and *parp2*. Both PARPs are activated by DNA damage caused by, example reactive oxygen species. Upon activation polymers of ADP-ribose are synthesized on a range of nuclear enzymes using NAD^+ as substrate. Here, we show that in plants stresses such as drought, high light and heat activate PARP causing NAD^+ breakdown and ATP consumption. When the PARP activity is reduced by means of chemical inhibitors or by gene silencing, cell death is inhibited and plants become tolerant to a broad range of abiotic stresses like high light, drought and heat. Plant lines with low poly(ADP-ribosyl)ation activity maintain under stress conditions their energy homeostasis by reducing NAD^+ breakdown and consequently energy consumption. The higher energy-use efficiency avoids the need for a too intense mitochondrial respiration and consequently reduces the formation of reactive oxygen species. From these results it can be concluded that breeding or engineering for a high energy-use efficiency under stress conditions is a valuable, but until today nearly unexploited, approach to enhance overall stress tolerance of crops.

Keywords: cell death, energy metabolism, mitochondrial respiration, NAD^+, poly(ADP-ribose) polymerase, stress tolerance.

Introduction

Over the past decades breeders have successfully improved the maximum attainable yield of the main field crops. These yield improvements are partly or, as in corn, primarily attributable to the enhancement of the overall stress tolerance (Duvick, 1997; Tollenaar and Lee, 2002; Tollenaar and Wu, 1999). Despite this steady progress, today still only 30–70% of the attainable yield is actually realized. This is due to unfavourable growth conditions such as nutrient shortage, cold nights, drought at the young seedling stage, high temperatures at flowering time, etc. This difference between the maximum attainable yield and the average farmer yield is referred to as the 'yield gap' (Dobermann *et al.*, 2003; Murrell and Childs, 2000). To narrow the yield gap the growth optimum of the crop for many growth parameters and their combinations should be broadened. Both breeding and biotechnology are nowadays exploited to reduce the yield gap. In breeding, varieties are being selected by their performance in different geographical locations over several years, whereas in biotechnology the recent insights into the mode of action of numerous genes are being exploited to engineer stress tolerance. By enhancing the tolerance to multiple stresses, plants become less sensitive to adverse environmental changes and this broadens indirectly the growth optimum. Because most types of stresses induce the production of reactive oxygen species, the overproduction of detoxification enzymes such as superoxide dismutases was one of the first approaches that have been evaluated to enhance the overall stress tolerance in plants (Alscher *et al.*, 2002). In addition, the overproduction of compatible solutes such as trehalose (Garg *et al.*, 2002; Jang *et al.*, 2003) and glycine betaine (Chen and Murata, 2002) result in stress-protective effects, which is due to the ability to scavenge reactive oxygen species besides their chaperon-like activity. Most of the current stress research is focused on understanding the stress signalling pathways. Many of the transcription factors (Chinnusamy *et al.*, 2003; Dubouzet *et al.*, 2003; Gilmour *et al.*, 2000; Kasuga *et al.*, 1999; Knight and Knight, 2001; Xiong *et al.*, 2002; Zhu, 2001) and more recently components of the mitogen-activated protein kinase signal transduction pathway (Meskiene and Hirt, 2000; Moon *et al.*,

2003a; Shou *et al.*, 2004) have been identified. Numerous reports show that stress tolerance may be improved by overexpressing these components. For comprehensive overviews about the current state of biotechnology in stress research we refer to Datta (2002) and Wang *et al.* (2003). A very different but promising approach to promote broad stress tolerance is by blocking stress-induced cell death. By overexpression of animal cell death suppressors such as Bax-inhibitors, Bcl2, Bcl-X_L, DAD-1, and IAP plants with improved tolerance to both necrotrophic pathogens and abiotic stresses such as UV, cold, salt and reactive oxygen species have been obtained (Awada *et al.*, 2003; Dickman *et al.*, 2001; Lincoln *et al.*, 2002; Moon *et al.*, 2003b; del Pozo and Lam, 2003; Qiao *et al.*, 2002). The same is true when the plant-derived anti-apoptotic genes such as BI-1, nucleoside diphosphate kinase 2 and DAD-1 were overexpressed in plants (Bolduc *et al.*, 2003; Hoeberichts and Woltering, 2003; Kawai-Yamada *et al.*, 2001; Matsumura *et al.*, 2003).

In this paper we describe a very different strategy to broaden stress tolerance in plants, by maintaining energy homeostasis under stress conditions. Today, there is no clear picture about the influence of stresses on energy metabolism, but in general stresses cause high energy consumption and enhance the respiration with a linked production of reactive oxygen species (Rizhsky *et al.*, 2002; Tiwari *et al.*, 2002). When the stresses are extreme or persistent an energy threshold is reached at which the damage caused by the stress can no longer be repaired, and the cell, tissue or ultimately the whole plant will die. This would predict that when stress-induced energy consumption could be reduced by enhancing the energy-use efficiency, the plant could overcome peak stresses or have the opportunity to acclimate to moderate but persistent stresses. The major stress-induced energy-consuming processes in plants are not well known, but in animals the stress-induced activation of poly(ADP-ribose) polymerase is the main cause of energy depletion. The enzymes PARP1 [Poly(ADP-Ribose) Polymerase-1], and to a lesser extent PARP2 [Poly(ADP-Ribose) Polymerase-2], are mainly responsible for the stress-induced poly(ADP-ribosyl)ation activity in animals. Both PARPs are activated by DNA damage caused by, example radicals (Virág and Szabó, 2002). Upon activation polymers of ADP-ribose are synthesized on a range of nuclear enzymes using NAD^+ as substrate (Bakondi *et al.*, 2002). Overactivation of PARP, as occurs in ischaemia, inflammation and neural diseases as Alzheimer's, causes a rapid breakdown of the NAD^+ pool (Du *et al.*, 2003; Klaidman *et al.*, 2003; Nakamura *et al.*, 2003; Virág and Szabó, 2002). As a consequence resynthesis of NAD^+ is stimulated whereby three (NAD^+-salvage pathway) to five (*de novo* synthesis) molecules of ATP are used for each molecule of NAD^+. In this way the cellular ATP is depleted which leads to necrotic cell death (Filipovic *et al.*, 1999; Ha and Snyder, 1999; Virág and Szabó, 2002). PARP1 is a 116-kDa nuclear enzyme composed of three functional domains: an N-terminal DNA-binding domain containing two zinc-finger motifs, a central automodification domain and a C-terminal catalytic domain, which is the most conserved region between PARP1 and PARP2. The catalytic domain contains a very conserved block of 50 amino acids, referred to as the PARP signature. PARP2 is a 62-kDa enzyme and is also located in the nucleus. It is composed of an N-terminal DNA-binding domain, however, without zinc-fingers, and a C-terminal catalytic domain containing the PARP signature (Virág and Szabó, 2002).

In plants, PARP1 and PARP2 homologues are found with a very similar structure to their animal counterparts (Babiychuk *et al.*, 1998). Both PARPs are localized in the nucleus and are activated by DNA strand breaks (Babiychuk *et al.*, 1998; Chen *et al.*, 2003; Doucet-Chabeaud *et al.*, 2001; Puchta *et al.*, 1995). Experiments in Arabidopsis have shown that DNA strand breaks, caused by ionizing radiation or oxidative stress, induce rapid and massive accumulation of both *parp1* and *parp2* transcripts in all plant tissues. The *parp2* gene is also induced by different kinds of environmental stresses such as drought and heavy metals (Doucet-Chabeaud *et al.*, 2001). Amor *et al.* (1998) showed that in cultured soybean cells expressing antisense *parp2* mRNA, H_2O_2-induced cell is inhibited.

In this paper we provide evidence for a role of PARP in energy homeostasis and stress tolerance. Our data show that in plants strong stresses induce poly(ADP-ribosyl)ation-activity causing NAD^+ breakdown and an enhanced mitochondrial respiration. By reducing stress-induced poly(ADP-ribosyl)ation-activity NAD^+ breakdown is inhibited preventing high energy consumption. Under these conditions plants preserve their energy homeostasis and this without an overactivation of the mitochondrial respiration, avoiding the production of reactive oxygen species. In this way, plants with a lowered poly(ADP-ribosyl)ation activity appear tolerant to multiple stresses.

Results

PARP inhibitors enhance the tolerance of Brassica napus *hypocotyl explants to oxidative stress*

In animals poly(ADP-ribosyl)ation has a major role in DNA repair, gene expression, cell cycle regulation, recombination, and cell death. Except for yeast, PARP enzymes have been found in all studied eukaryotes.

As a first approach to decipher the role of PARP in plants, the effect of a small panel of chemical inhibitors on the growth of *Brassica napus* callus was studied. Three PARP-inhibitors were used: 3-methoxybenzamide (3MB), nicotinamide (NA), and isonicotinamide (INA). Animal PARP1 is inhibited at 1 mM concentrations with 92% by 3MB, 72% by NA and 51% by INA (Banasik *et al.*, 1992). Figure 1 shows the

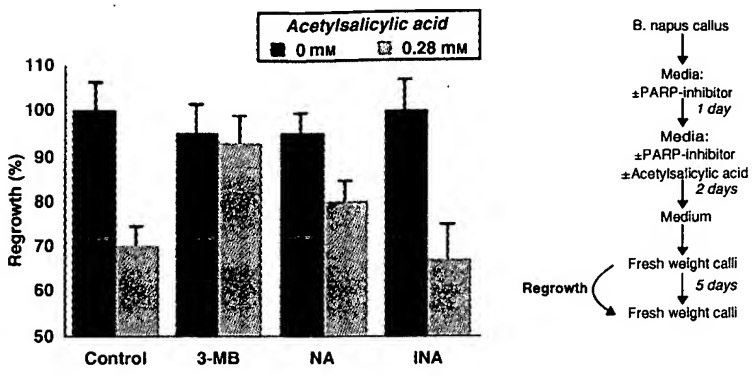

Figure 1. PARP inhibitors protect *Brassica napus* callus against oxidative stress. *Brassica napus* callus was incubated for 2 days on medium containing 0.28 mM acetylsalicylic acid. The regrowth of the callus was scored after another 5 days. The PARP inhibitors 3-methoxybenzamide (3-MB), nicotinamide (NA) and isonicotinamide (INA) were used at 1 mM concentrations. The regrowth of the non-treated callus (no PARP inhibitors, no acetylsalicylic acid) was set at 100%. Error bars indicate the standard error ($n = 3$).

layout and results of an experiment in which the impact of 3MB, NA and INA on the regrowth of *B. napus* callus was measured after 2 days of oxidative stress induced by acetylsalicylic acid (De Block and De Brouwer, 2002). When 3MB was used callus regrowth was hardly inhibited by oxidative stress. The weaker inhibitor NA had some protective effect, while the weakest inhibitor INA did not protect the tissue against acetylsalicylic acid. These observations indicate that PARP inhibitors protect explants in tissue culture against oxidative stress and disclose a relationship between PARP activity and stress sensitivity.

Overexpression of dsRNA-parp constructs enhances the overall stress tolerance of plants

As PARP inhibitors are not completely specific and potentially also inhibit other enzymes (Banasik *et al.*, 1992; Cosi, 2002; Southan and Szabó, 2003) a genetic approach was used to understand the role and function of PARP in stress tolerance. Arabidopsis and oilseed rape were transformed with dsRNA constructs containing the 5′-end of the Arabidopsis *AtParp1* or *AtParp2* genes in the stem structure (Waterhouse *et al.*, 1998, 2001). These constructs are indicated as *hpAtParp1* and *hpAtParp2* (hp stands for hairpin). Segregating populations for the hpRNA constructs obtained by selving or backcrossing of the heterozygous lines, were tested for tolerance to various stresses like drought, heat, and high light. The phenotype and growth of the transgenic plants were compared with their azygous counterparts. Of 50 lines per construct and plant species, 10% of the hpAtParp1 and 20% of the hpAtParp2 lines were very tolerant to the applied stresses, while another 20% of the hpAtParp1 and 30% of the hpAtParp2 lines was partly stress tolerant. Figure 2(a,b) shows representative phenotypes that were observed in stress experiments. The graph in Figure 2(d) depicts at the end of a drought experiment the fresh weight of the transgenic and azygous plants of 1:1 segregating Arabidopsis populations for the *hpAtParp* transgenes. The weight of the azygous plants was similar to these of the non-transgenic control. The plants transgenic for the *hpAtParp1* or *hpAtParp2* construct had on average 70% higher fresh weight than their azygous counterparts. Lines that were tolerant to drought were also tolerant to methyl viologen (Figure 3c). The frequencies with which stress tolerant lines were obtained indicate that downregulation of *parp2* is more effective than downregulation of *parp1*.

The impact of both *parp1* and *parp2* on stress tolerance was studied in an experiment with a similar set-up as outlined in Figure 2(b). By crossing a transgenic Arabidopsis line was made containing *hpAtParp1* and *hpAtParp2* loci that each provided stress tolerance in the parental lines (Figure 2b–d). The stress tolerance of the (hpAtParp1 + hpAtParp2) line was compared with the parental lines. The graph in Figure 2(e) shows that overexpression of both the *hpAtParp1* and *hpAtParp2* genes results in a higher stress tolerance than overexpression of only one of the *hpAtParp* genes.

This finding was further supported by studies with Arabidopsis lines carrying an hpRNA construct targeting the sequence of the *AtParp2* catalytic domain around the PARP signature (Amé *et al.*, 1999; Babiychuk *et al.*, 1998). This region has an overall sequence similarity of 86% between *AtParp1* and *AtParp2*, with a block of 24 bp with 100% sequence similarity at the PARP-signature. This construct, indicated as *hpAtParp2(signature)*, would allow silencing of both *AtParp1* and *AtParp2* (Holzberg *et al.*, 2002). Up to 40% of the hpAtParp2(signature) lines were tolerant to high light stress, while this was only 10% of the hpAtParp1 and 20% of the hpAtParp2 lines.

The stress-tolerant hpParp-lines have under stress conditions a reduced poly(ADP-ribosyl)ation activity

The activity of poly(ADP-ribose) polymerase results in the poly(ADP-ribosyl)ation (PAR) of nuclear proteins (Affar *et al.*, 1998; Bakondi *et al.*, 2002). This allowed us to test to what extent the observed stress tolerance correlates with the PAR activity. During high light stress an increase in poly (ADP-ribosyl)ated proteins was detected in the nuclei of wild-type Arabidopsis plants with a peak 4–6 h after start (Figure 3a). The hpAtParp1, hpAtParp2 and hpAtPARP2

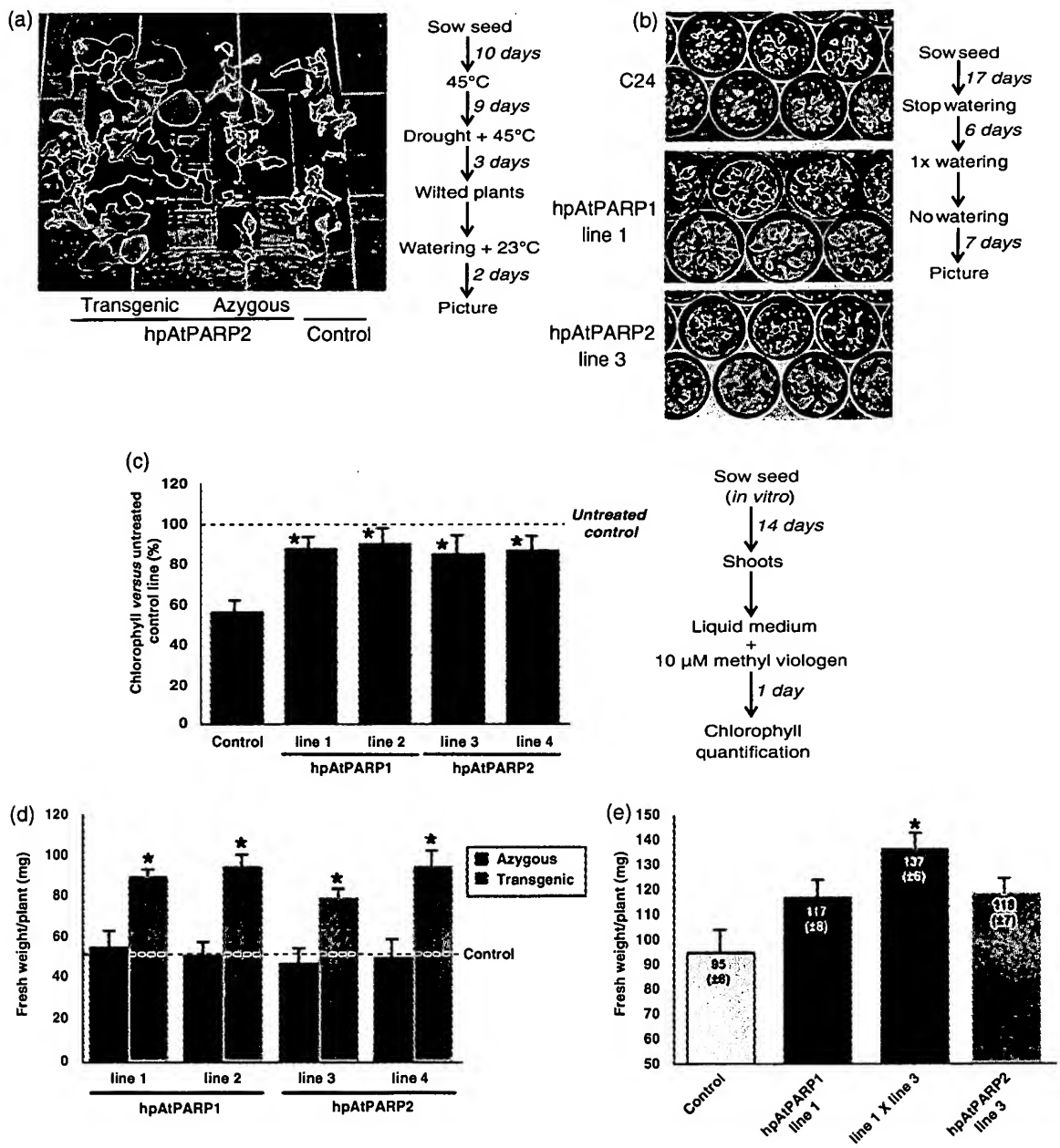

Figure 2. Overexpression of the *hpAtParp* constructs enhances the stress tolerance of plants.
(a) Phenotypes of a control and a 3 transgenic:1 azygous segregating *Brassica napus* hpAtParp2 line at the end of a stress experiment where heat and drought had been combined.
(b) Phenotypes of *Arabidopsis thaliana* cv. C24 lines at the end of a drought stress experiment.
(c) Chlorophyll content of *A. thaliana* cv. C24 untransformed control and homozygous hpAtParp lines treated with 10 µM methyl viologen for 1 day.
(d) Fresh weight of *Arabidopsis thaliana* cv. C24 plants from a 1 transgenic:1 azygous segregating hpAtParp1 and hpAtParp2 lines at the end of a drought stress experiment as outlined in Figure 2(b). The segregating lines for the *hpAtParp* constructs were obtained by backcrossing hemizygous hpAtParp plants with wild-type plants.
(e) The fresh weight of *Arabidoposis thaliana* cv. C24 plants at the end of a drought experiment as outlined in Figure 2(b). The line containing both the *hpAtParp1* and *hpAtParp2* constructs was obtained by crossing the homozygous hpAtParp1 line 1 with the homozygous hpAtParp2 line 3 (b–d). The numbers in the bars are the mean and the standard error. The hpAtParp lines 1 to 4 are the same in (b–e). The error bars indicate the standard error ($n = 3$). *Significant difference at $P = 0.01$.

(signature) lines that were scored as stress tolerant (Figures 2b–d and 5c–e) had low PAR activities, while the control and stress sensitive lines had high PAR activities, especially when stressed by high light (Figure 3b,c). These data strongly argue that the enhanced stress tolerance of the hpAtParp lines correlates with a low PAR activity at stress conditions.

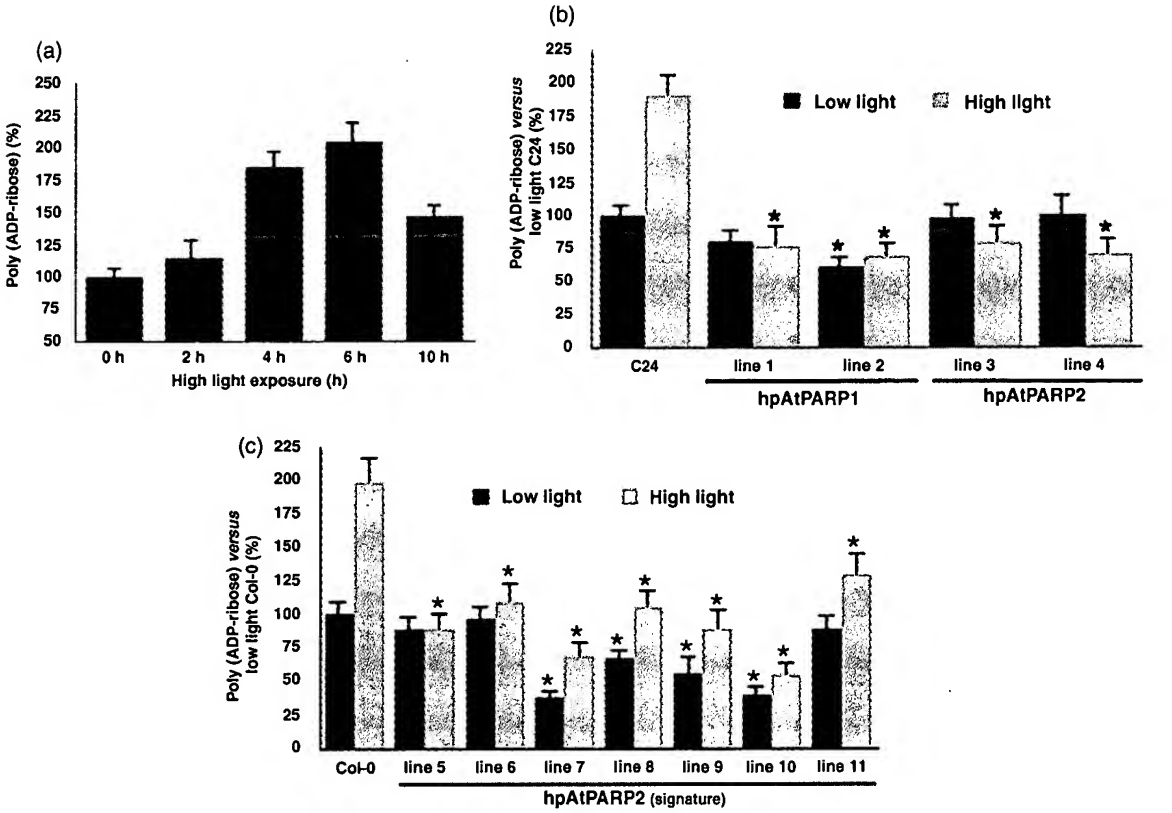

Figure 3. The hpAtParp lines have a reduced poly(ADP-ribosyl)ation activity (PAR).
(a) *Arabidopsis thaliana* cv. Col-0 was stressed by high light. Samples were taken at various time points and the PAR activity was determined.
(b) PAR activities of *Arabidopsis thaliana* cv. C24 wild-type and stress-tolerant hpAtParp1 or hpAtParp2 lines (Figure 2b–d) stressed by high light.
(c) PAR activities of *Arabidopsis thaliana* cv. Col-0 lines stressed by high light. The lines are transgenic for the *hpAtParp2(signature)* construct and have different tolerance levels to high light stress (Figure 5c–e): lines 6 and 11 are sensitive; lines 7 and 10 are tolerant; lines 8 and 9 have an intermediate tolerance. Low light (30 μEinstein m^{-2} sec^{-1}); high light (220 μEinstein m^{-2} sec^{-1}). The error bars indicate the standard error ($n = 3$). *Low and high light mean values are statistically different from the control at $P = 0.01$.

Inhibition of PARP reduces stress-induced cell death

In animals activation of PARP1 results in NAD^+ breakdown, ATP overconsumption and finally in energy depletion causing necrotic cell death, whereas inhibition of PARP activity leads to maintenance of the NAD^+ and ATP levels under stress conditions and a reduced cell death (Filipovic *et al.*, 1999; Ha and Snyder, 1999; Virág and Szabó, 2002). We also tested whether in plants inhibition of PARP would reduce stress-induced cell death. Both programmed and necrotic cell death are characterized by the degradation of nuclear DNA and can be visualized with the TUNEL assay that labels the DNA breaks (Gavrieli *et al.*, 1992). Figure 4 shows the result of an *in situ* TUNEL assay carried out on the top ends of *B. napus* hypocotyl explants incubated for 1 day in medium containing acetylsalicylic acid. Numerous nuclei were labelled in the cortex tissue of explants derived from control or azygous plants, while only few nuclei were labelled in explants derived from stress-tolerant transgenic hpAtParp plants. Similar results were obtained by treating explants derived from non-transgenic plants with the PARP-inhibitor 3-methoxybenzamide 1 day before and during the 1-day incubation in acetylsalicylic acid-containing medium. These data indicate that in plants inhibition of PARP protects the cells against stress-induced cell death (necrotic and/or programmed).

Inhibition of PARP prevents energy overconsumption in stress conditions

The above similarities between the role of PARP in animals and plants prompted us to investigate further parallels. We therefore tested whether the hpAtParp lines showed altered energy housekeeping. To this end, *B. napus* hypocotyl explants were cultured on medium containing 0.06 M glucose as carbon source. Glucose is a suboptimal carbon source for *in vitro* cultured *B. napus* hypocotyl explants, and

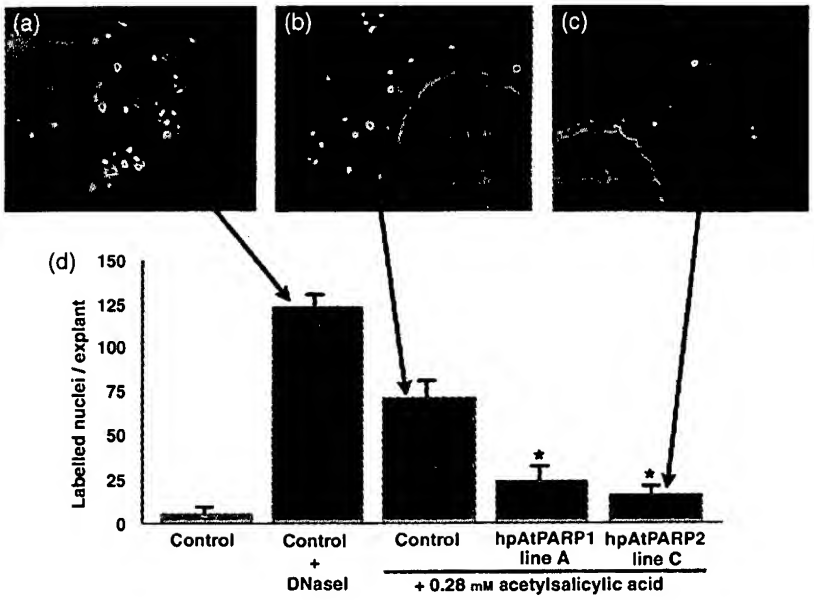

Figure 4. Stress-induced cell death is inhibited in the hpAtParp lines. TUNEL assay carried out on the top ends of 5-day-cultured *Brassica napus* hypocotyl explants.
(a) Control explants treated for 1 h at 37°C with 40 U ml^{-1} DNaseI. Most nuclei are labelled.
(b) Explants from a non-transgenic control incubated for 1 day in medium containing 0.28 mM acetylsalicylic acid.
(c) Explants from an hpAtParp2 line incubated for 1 day in medium containing 0.28 mM acetylsalicylic acid. The red arrows in (a–c) indicate examples of labelled nuclei.
(d) Quantification of the number of labelled nuclei/explant. The nuclei that were labelled in the cortex tissue of the explants were counted. In each experiment 50 explants/line and treatment were scored. The lines A and C correspond with lines A and C of Table 1. The error bars indicate the standard error ($n = 3$). *Mean values are statistically different from the acetylsalicylic acid treated control at $P = 0.01$.

concentrations higher than 0.1 M glucose have to be used to allow a good callus induction and growth. Figure 5(a) shows 3-week-old hypocotyl explants that were cultured on medium containing 0.06 M glucose. The explants of the control line are necrotic and have a poor callus formation, while the explants of the hpAtParp2 lines are still green and form vigorous callus. This is further illustrated in the graph of Figure 5(b) that shows the phenotype and weight of the hypocotyl explants of stress-sensitive and -tolerant hpAtParp lines that were cultured for 3 weeks on 0.06 M glucose. The stress-tolerant hpAtParp lines produced more callus and the hypocotyl explants did not become necrotic. However, when the seeds were germinated on medium containing 2–6% glucose no difference in seedling growth could be measured between the hpAtParp lines and the non-transgenic control line. This indicates that the differential callus formation and survival of the explants on glucose medium between the stress-tolerant hpAtParp lines and the control line is not due to an altered sugar sensing. Similar seedling-growth experiments with the Arabidopsis hpAtParp lines confirmed that downregulation of the *parp* genes does not alter sugar sensing. In summary, the above experiments suggest that reducing the PAR activity results in higher energy efficiency under stress conditions.

To test whether energy homeostasis is the basis of the stress tolerance of the hpAtParp lines, the total NAD^++NADH and ATP content were quantified under control and stress conditions. A non-transgenic control and four hpAtParp2(signature) Arabidopsis lines were stressed by high light. The four hpAtParp2(signature) lines had different levels of stress tolerance towards high light going from a tolerance comparable to the non-transgenic control line to a high stress tolerance (Figure 5e). The graph in Figure 5(c) shows that when the lines were more stress tolerant the total NAD^++NADH content was less affected by stress. This is also reflected in the ATP content that in the stress tolerant lines even increased by stress. This implies that energy homeostasis is at the basis of the observed stress tolerance.

Preventing energy overconsumption allows a normal mitochondrial respiration

Most stresses interfere with a normal mitochondrial function. This interference results in a high radical production that causes cell damage. When *B. napus* hypocotyl explants were incubated in medium containing 0.28 mM acetylsalicylic acid only a minor increase in superoxide production was measured in the explants derived from the hpAtParp lines: about 3–8% increase in the hpAtParp lines versus about 167% in the controls (Table 1). This pointed to an efficient mitochondrial electron transport in the hpAtParp lines that was further confirmed by the high ratio of moles ATP present in the explants to the respiration rate.

The high energy status, the efficient cellular respiration and the low radical production indicate that there is under stress lower energy consumption and by this a lower respiration rate in the hpAtParp lines when compared with the controls. This prediction was tested by measuring the capacity of the lines to reduce 2,3,5-triphenyltetrazolium-chloride (TTC). TTC is reduced by the mitochondrial electron transport system to an insoluble formazan that can be extracted from the cells and tissues by ethanol and subsequently quantified spectrophotometrically (De Block and De Brouwer, 2002). Figure 5(d) shows the results of a TTC assay on high light-stressed Arabidopsis lines. The stress-tolerant

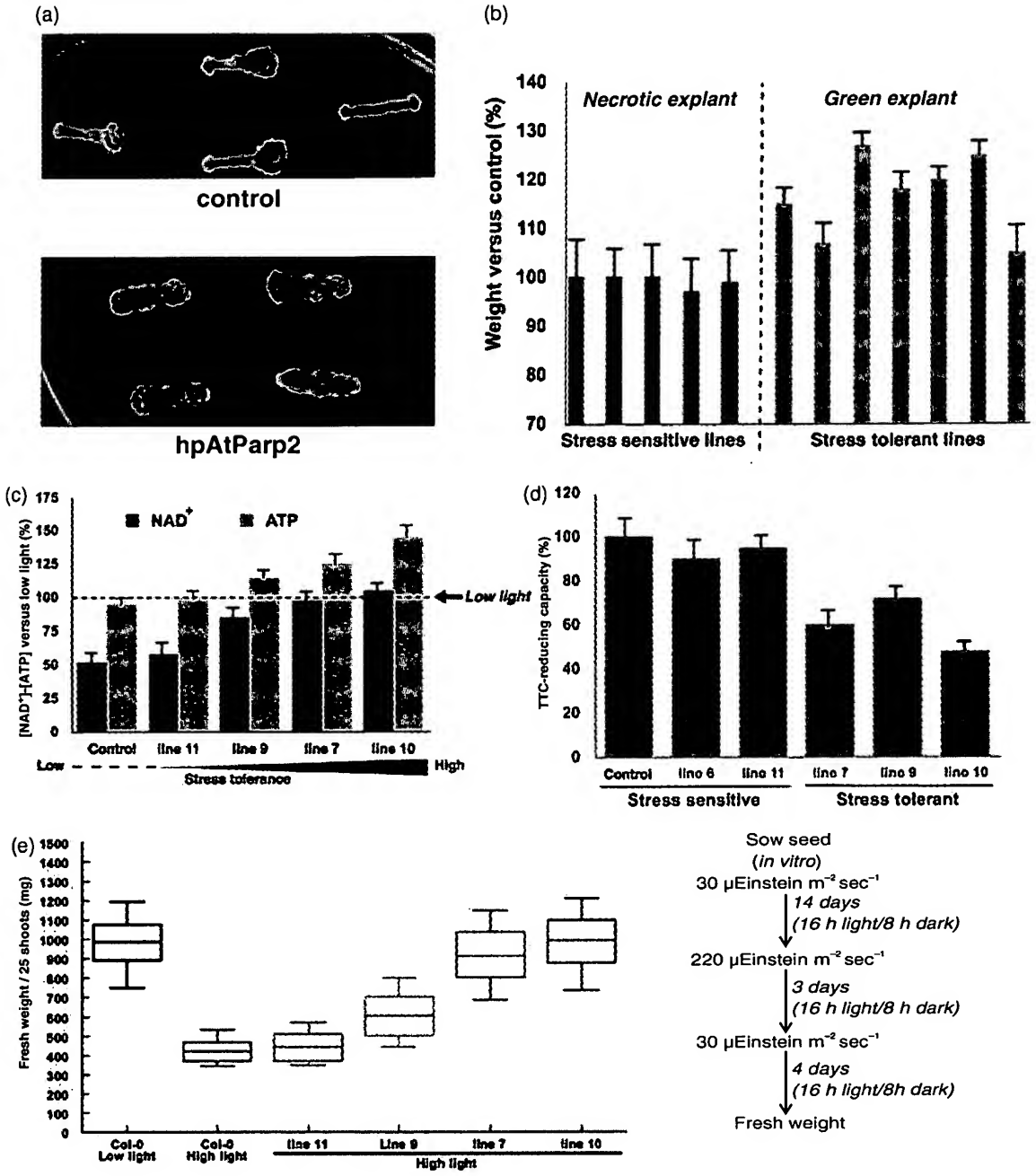

Figure 5. The hpAtParp lines maintain their energy homeostasis under stress conditions.

(a) Phenotypes of *Brassica napus* hypocotyl explants cultured for 2 weeks on callus inducing medium containing 0.06 M glucose as carbon source. The red arrow indicates a necrotic explant.

(b) Fresh weight of *B. napus* hypocotyl explants cultured for 2 weeks on callus inducing medium containing 0.06 M glucose as carbon source. The callus weight of the non-transgenic control line was set at 100%. The error bars indicate the standard error ($n = 3$).

(c) NAD^++NADH and ATP contents of control and hpAtParp2(signature) *Arabidopsis thaliana* cv. Col-0 lines that have been stressed for 24 h by high light. The NAD^++NADH and ATP contents of the non-stressed control line were set at 100% and are indicated by the dashed line 'low light' (30 µEinstein m^{-2} sec^{-1}). The lines are sorted from low to high stress tolerance as scored in high light experiments (e).

(d) Quantification of mitochondrial electron transport by measuring TTC reduction. TTC-reducing capacity of control and hpAtParp2(signature) *A. thaliana* cv. Col-0 lines. The plants were stressed for 24 h by high light. Error bars indicate standard error ($n = 3$).

(e) Tolerance or hpAtParp2(signature) lines to high light stress. High light stress was applied as described in the scheme. Fresh weight was determined per 25 shoots. For each experiment 100 shoots per line and condition were used. The lines in the boxes represent the mean, the lower and upper edges of the box the 25th and 75th percentiles, and the whiskers the 10th and 90th percentiles.

Table 1 Influence of acetylsalicylic acid on energy and radical production of 5 days cultured *B. napus* hypocotyl explants

Line	Stress	Moles ATP[a] / mg consumed O_2[b] (SE)[c]	% superoxide production versus control (SE)[c]
Control	None	4.19×10^{-6} ($\pm 0.25 \times 10^{-6}$)	–
	ASA[d]	2.85×10^{-6} ($\pm 0.25 \times 10^{-6}$)	+167 (±13)
hpAtParp1 (line A)	None	3.99×10^{-6} ($\pm 0.21 \times 10^{-6}$)	–
	ASA[d]	4.04×10^{-6} ($\pm 0.28 \times 10^{-6}$)	+8 (±2)
hpAtParp1 (line B)	None	4.10×10^{-6} ($\pm 0.19 \times 10^{-6}$)	–
	ASA[d]	4.25×10^{-6} ($\pm 0.27 \times 10^{-6}$)	+4 (±2)
hpAtParp2 (line C)	None	4.07×10^{-6} ($\pm 0.24 \times 10^{-6}$)	–
	ASA[d]	4.22×10^{-6} ($\pm 0.33 \times 10^{-6}$)	+4 (±2)
hpAtParp2 (line D)	None	4.15×10^{-6} ($\pm 0.22 \times 10^{-6}$)	–
	ASA[d]	4.28×10^{-6} ($\pm 0.25 \times 10^{-6}$)	+3 (±2)

[a] g^{-1} fresh weight.
[b] h^{-1} g^{-1} fresh weight.
[c] SE ($n = 3$).
[d] Explants were incubated for 1 day in medium containing 0.28 mM acetylsalicylic acid.

lines have a lower TTC-reducing capacity/respiration than the stress-sensitive lines.

Discussion

In this paper we described the utility of reducing poly(ADP-ribosyl)ation activity to engineer broad stress tolerance in plants. Our data show that: First, plants with reduced PAR-activity are tolerant to a broad range of stresses. Secondly, stress tolerance is obtained by maintaining energy homeostasis. Thirdly, energy homeostasis is maintained by reducing stress-induced energy consumption by preventing NAD^+ breakdown. This results in higher energy-use efficiency. Fourthly, reducing energy consumption avoids a too intense mitochondrial respiration and consequently prevents the formation of reactive oxygen species.

These data point to the importance of the cellular energy metabolism in relation to stress in plants. In animals energy depletion due to mitochondrial dysfunction and/or by energy consuming processes may lead to both programmed and necrotic cell death (Ricci *et al.*, 2003). When the ATP content of a cell drops below a certain threshold mitochondrial permeability transition pores are formed. This causes the release of cell death initiators and the breakdown of ATP by the mitochondrial ATPase. Free radicals and reactive oxygen species may damage the mitochondria directly by attacking the mitochondrial enzymes and the electron transport chain and by opening the mitochondrial permeability transition pores. In this way the energy production process is disrupted. The combination of high energy consumption with a poor ATP production will finally result in cell death. The theory that PARP upon activation is one of the major energy consumers and that overactivation of PARP results in cell death by energy depletion has been confirmed by numerous studies. In most, but not all studies, it has been proved that pharmacological inhibition or genetic inactivation of PARP prevents energy depletion and necrotic cell death (Filipovic *et al.*, 1999; Ha and Snyder, 1999; Virág and Szabó, 2002). It has also been observed that even a moderate PARP activation may decrease the cellular NAD^+ content sufficient to compromise the cellular energy status. This does not cause cell death but a dysfunction of the cell. In these cases pharmacological inhibition of PARP improves cellular energetics and restores cell function (Virág and Szabó, 2002).

Although the knowledge about cellular energetics in regard to stress is less elaborated in plants, it is known that oxidative stress increases cellular respiration, interferes with mitochondrial energy production and causes ATP depletion (Tiwari *et al.*, 2002). In this paper we showed that in plants PARP is a major energy consumer under stress conditions, and that both pharmacological and genetic inhibition of PARP reduces stress-induced energy consumption, protects plants against stress or enables plants to recover from stress injury, and prevents cell death. As in animals the PARP-induced cell death is probably necrotic. Although the nuclei of stressed explants could be labelled by means of the TUNEL assay (Figure 4), that allows to detect both necrotic and programmed cell death, the typical hallmarks for programmed cell death as DNA laddering and cell shrinkage could not be observed. While in animals PARP1 is more abundant than PARP2 and is responsible for 90% of the total PARP activity, this is not the case in plants. Doucet-Chabeaud *et al.* (2001) showed that in Arabidopsis both *parp1* and *parp2* are equally induced by DNA breaks, while stresses as dehydration and heavy metals mainly induce *parp2*. This is in accordance with our results. First, stress tolerance is obtained by reducing PARP1 or PARP2 activity. Secondly, in general, downregulation of PARP2 is more effective for obtaining stress tolerance.

In our study we mainly elucidated the 'pathological' site of PARP. The biological function of PARP in plants is not yet clear, but experimental evidence indicates that PARP is involved in DNA repair also in plants (Puchta *et al.*, 1995).

The presence of *parp* genes in all eukaryotes except yeast and the conserved structure of the enzymes points to their importance for a normal functioning of the organism. Mice knockout lines for *parp1* or *parp2* are defective in DNA excision repair and are hypersensitive to alkylating agents (Schreiber *et al.*, 2002; Trucco *et al.*, 1998) while double knockouts for both *parp1* and *parp2* are not viable (Ménissier de Murcia *et al.*, 2003). To date, we did not find in the hpAtParp-lines any negative effect of downregulation, nor in growth or seed set. We tested the most stress-tolerant Arabidopsis lines for sensitivity to the alkylating agent EMS, but no higher mutation frequency versus the non-transgenic control line was found (data not shown). However, as can be seen in Figure 3, in the stress-tolerant lines the poly(ADP-ribosyl)ation activity is not completely downregulated. Probably, the remnant activity still allows a normal DNA repair as observed in animals using pharmacological inhibitors (Virág and Szabó, 2002).

In conclusion, reducing poly(ADP-ribosyl)ation activity in plants confers tolerance to a broad range of abiotic stresses, by maintaining energy homeostasis. These findings indicate that breeding or engineering for higher energy-use efficiency could be a valuable approach to enhance overall stress tolerance in crops.

Experimental procedures

Plasmid constructs

The *hpAtParp1* construct consists from 5′ to 3′: the cauliflower 35S promoter (Odell *et al.*, 1985) – DNA fragment containing nucleotides 429–1469 of the *AtParp1* cDNA clone – DNA fragment containing nucleotides 428–955 of the *AtParp1* cDNA clone in inverted orientation – 3′ end polyadenylation signal of CaMV35S (Sanfacon *et al.*, 1991). *AtParp1* cDNA clone: GenBank Accession Z48243.

The *hpAtParp2* construct consists from 5′ to 3′: the cauliflower 35S promoter – DNA fragment containing nucleotides 190–1348 of the *AtParp2* cDNA clone – DNA fragment containing nucleotides 189–781 of the *AtParp2* cDNA clone in inverted orientation – 3′ end polyadenylation signal of CaMV35S. *AtParp2* cDNA clone: GenBank Accession AJ131705.

The *hpAtParp2(signature)* construct was made using the pHANNIBAL vector (Helliwell and Waterhouse, 2003). The DNA fragments containing the nucleotides 1572–1730 and 1571–1729 of the *AtParp2* cDNA clone, were cloned in direct inverted orientation.

The *AtParp1* and *AtParp2* cDNA clones were provided by S. Kushnir (Flemish Interuniversity Institute for Biotechnology, Gent, Belgium).

All plasmids constructs contained the *bar* gene (De Block *et al.*, 1987) under the control of the promoter from the Arabidopsis actin-2 gene (Kandasamy *et al.*, 2002) as selectable marker gene.

In vitro *culture of Brassica napus*

The *in vitro* culture of hypocotyl explants was mainly carried out as described (De Block *et al.*, 1989).

In each experiment 150 explants derived from 30 to 40 seedlings were used per line and per condition.

Stress assays

Seeds were sterilized with bleach containing 6% active chlorine and subsequently pre-germinated for 1 day in sterile tap water. Pre-germination reduces the impact of seed vigour on early seedling growth.

The transgenic and azygous plants in segregating populations for the *p35S:hpAtPARP-pact:bar* construct were identified by testing the plants for the presence of the enzyme phosphinothricin acetyl transferase using the Trait LL Leaf/Seed Test Kit of Strategic Diagnostics Inc. (Newark, NJ, USA).

Arabidoposis – high light stress. Plants were grown *in vitro* at 30 μEinstein m^{-2} sec^{-1} (low light) for 2 weeks (16 h light and 8 h dark). The high light stress was applied by transferring the plants to 200–250 μEinstein m^{-2} sec^{-1}.

For quantification of NAD^+, ATP, respiration and radical production, about 60 plants (1–2 g fresh weight) were used per line and condition.

When the tolerance to high light stress on plant growth was studied, 100 plants per line were treated for 3 days (16 h light and 8 h dark) at 200–250 μEinstein m^{-2} sec^{-1}. After the high light treatment the plants were grown for another 4 days. The fresh weight was determined per 25 plants.

Arabidoposis – drought stress. Seedlings were grown for 7–8 days *in vitro* (to obtain homogenous populations) after which they were transferred to flats with 51 pots containing sandy soil. Eight to 9 days after transfer water was withheld for 6 days after which 20 ml water/plant was added once. Results were scored when the azygous and control plants turned yellow, this took another 7–10 days.

Arabidoposis – methyl viologen treatment. In this assay seeds from lines homozygous for the transgene were used as starting material. The plants were grown *in vitro* at 30–50 μEinstein m^{-2} sec^{-1} for 2 weeks (16 h light and 8 h dark). The shoots were harvested in batches of 500 mg (roots were removed) and flooded in Petri dishes containing liquid medium with 10 μM methyl viologen and 0.1% Tween 20. The Petri dishes were incubated in the light (50 μEinstein m^{-2} sec^{-1}; 6 h light and 8 h dark) for 24 h. Before extraction the shoots were frozen at −70°C and subsequently thawed at 40°C. The chlorophyll was extracted with ethanol: 50 ml ethanol for 500 mg shoots. The OD of the extracts was measured at 663 nm.

Brassica napus – *heat and drought stress.* After pre-germination the seeds were transferred to 12 cm pots. After 10 days the seedlings were transferred to 45°C. Nine to 10 days later water was withheld for 3 days after which the plants were severely wilted. The plants were transferred to 23°C and watered normally. A few days later the damage to the plants was scored.

Whole mount TUNEL assay

Fixation was made with formalin acetic acid for 4 h. The samples were dehydrated with 50% (1 h) and 70% ethanol (1 h; refresh and store at −20°C). The tissues and cells were permeabilized with 0.3% Triton X100 (10 min) and 40 μg ml^{-1} proteinase K (15 min at room temperature). All the buffers contained 0.1% Tween 20 (Sigma, St Louis, MO, USA). DNA breaks were detected by using the '*In Situ* Detection Kit, Fluorescein' from Roche (Mannheim, Germany). For a

positive control fixed and permeabilized explants were treated for 1 h at 37°C with 40 U ml^{-1} DNaseI.

Labelling was evaluated by means of fluorescence microscopy (Axioplan 2; Zeiss, Jena, Germany). The nuclei that were labelled in the cortex of the explants were counted: 50 explants per line and condition.

ATP, total NAD^++NADH and superoxide content

ATP quantification was carried out as described (Rawyler *et al.*, 1999). Total NAD^++NADH was quantified as described (Filipovic *et al.*, 1999; Karp *et al.*, 1983). The formation of superoxides was quantified by measuring the reduction of 3'-{1-[phenylamino-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) (=XTT) as described (De Block and De Brouwer, 2002).

Poly(ADP-ribosyl)ation activity: immunological detection of poly(ADP-ribose)

For the isolation of plant nuclei and nuclear protein extraction the CelLytic PN kit of Sigma was used. Of each sample 0.5–1 μg of nuclear protein was spotted on a Hybond-C nitrocellulose membrane (Amersham, Buckinghamshire, UK). The poly(ADP-ribosyl)ated proteins were detected by means of anti-PAR antibodies (Calbiochem, San Diego, CA, USA) as primary antibody and anti-rabbit IgG alkaline phosphate conjugate (Sigma) as secondary antibody. Western Blue stabilized substrate for alkaline phosphatase (Promega, Madison, WI, USA) was used for staining. The image analysing software 'ImageJ 1.32j' (http://rsb.info.nih.gov/ij) was used to quantify the total intensity of the spots.

Oxygen consumption and TTC-reducing capacity

For measuring oxygen consumption a Clark polarographic electrode was used (Cyberscan DO310; Eutech Instruments, Singapore). The capacity of the plant lines to reduce 2,3,5-triphenyltetrazoliumchloride (=TTC) was quantified as described (De Block and De Brouwer, 2002).

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References

Affar, E.B., Duriez, P.J., Shah, R.G., Sallmann, F.R., Bourassa, S., Küpper, J.-H., Bürkle, A. and Poirier, G.G. (1998) Immunodot blot method for the detection of poly(ADP-ribose) synthesized in vitro and in vivo. *Anal. Biochem.* **259**, 280–283.

Alscher, R.G., Erturk, N. and Heath, L.S. (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J. Exp. Bot.* **53**, 1331–1341.

Amé, J.-C., Rolli, V., Schreiber, V., Niedergang, C., Apiou, F., Decker, P., Muller, S., Höger, T., Ménissier-de Murcia, J. and de Murcia, G. (1999) PARP-2, a novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase. *J. Biol. Chem.* **274**, 17860–17868.

Amor, Y., Babiychuk, E., Inzé, D. and Levine, A. (1998) The involvement of poly(ADP-ribose) polymerase in the oxidative stress responses in plants. *FEBS Lett.* **440**, 1–7.

Awada, T., Dunigan, D.D. and Dickman, M.B. (2003) Animal anti-apoptotic genes ameliorate the loss of turgor in water-stressed transgenic tobacco. *Can. J. Plant Sci.* **83**, 499–506.

Babiychuk, E., Cottrill, P.B., Storozhenko, S., Fuangthong, M., Chen, Y., O'Farrell, M.K., Van Montagu, M., Inzé, D. and Kushnir, S. (1998) Higher plants possess two structurally different poly(ADP-ribose) polymerases. *Plant J.* **15**, 635–645.

Bakondi, E., Bai, P., Szabó, É., Hunyadi, J., Gergely, P., Szabó, C. and Virág, L. (2002) Detection of poly(ADP-ribose) polymerase activation in oxidatively stressed cells and tissues using biotinylated NAD substrate. *J. Histochem. Cytochem.* **50**, 91–98.

Banasik, M., Komura, H., Shimoyama, M. and Ueda, K. (1992) Specific inhibitors of poly(ADP-ribose) synthetase and mono (ADP-ribosyl) transferase. *J. Biol. Chem.* **267**, 1569–1575.

Bolduc, N., Ouellet, M., Pitre, F. and Brisson, L.F. (2003) Molecular characterization of two plant BI-1 homologues which suppress Bax-induced apoptosis in human 293 cells. *Planta*, **216**, 377–386.

Chen, T.H.H. and Murata, N. (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol.* **5**, 250–257.

Chen, I.-P., Haehnel, U., Altschmied, L., Schubert, I. and Puchta, H. (2003) The transcriptional response of Arabidopsis to genotoxic stress – a high-density colony array study (HDCA). *Plant J.* **35**, 771–786.

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.-H., Hong, X., Agarwal, M. and Zhu, J.-K. (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. *Genes Dev.* **17**, 1043–1054.

Cosi, C. (2002) New inhibitors of poly(ADP-ribose) polymerase and their potential therapeutic targets. *Expert. Opin. Ther. Patents*, **12**, 1047–1071.

Datta, S.K. (2002) Recent developments in transgenics for abiotic stress tolerance in rice. *JIRCAS Working Report*, ?? 43–53.

De Block, M. and De Brouwer, D. (2002) A simple and robust *in vitro* assay to quantify the vigour of oilseed rape lines and hybrids. *Plant Physiol. Biochem.* **40**, 845–852.

De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gosselé, V., Movva, N.R., Thompson, C., Van Montagu, M. and Leemans, J. (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *EMBO J.* **6**, 2513–2518.

De Block, M., De Brouwer, D. and Tenning, P. (1989) Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the bar and neo genes in the transgenic plants. *Plant Physiol.* **91**, 694–701.

Dickman, M.B., Park, Y.K., Oltersdorf, T., Li, W., Clemente, T. and French, R. (2001) Abrogation of disease development in plants expressing animal antiapoptotic genes. *Proc. Natl Acad. Sci. USA*, **98**, 6957–6962.

Dobermann, A., Arkebauer, T., Cassman, K. *et al.* (2003) Understanding corn yield potential in different environments. *Annual Report to the Fluid Fertilizer Foundation on grant 'Yield potential and optimal soil productivity in irrigated corn systems of the north-central USA'*, 1–16.

Doucet-Chabeaud, G., Godon, C., Brutesco, C., de Murcia, G. and Kazmaier, M. (2001) Ionising radiation induces the expression of PARP-1 and PARP-2 genes in Arabidopsis. *Mol. Genet. Genomics*, **265**, 954–963.

Du, L., Zhang, X., Han, Y.Y., Burke, N.A., Kochanek, P.M., Watkins, S.C., Graham, S.H., Carcillo, J.A., Szabó, C. and Clark, R.S.B. (2003) Intra-mitochondrial poly(ADP-ribosylation) contributes to

NAD+ depletion and cell death induced by oxidative stress. *J. Biol. Chem.* **278**, 18426–18433.

Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G., Miura, S., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2003) OsDREB genes in rice, *Oryza sativa* L, encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *Plant J.* **33**, 751–763.

Duvick, D.N. (1997) What is yield? In: *Developing Drought and Low N-tolerant Maize. Proceedings of a Symposium, March 25–29, 1996* (Edmeades, G.O., Banziger, B., Mickelson, H.R. and Peña-Valdivia, C.B., eds). Mexico: CIMMYT, El Batan, pp. 332–335.

Filipovic, D.M., Meng, X. and Reeves, W.B. (1999) Inhibition of PARP prevents oxidant-induced necrosis but not apoptosis in LLC-PK1 cells. *Am. J. Physiol.* **277**, F428–F436.

Garg, A.K., Kim, J.-K., Owens, T.G., Ranwala, A.P., Do Choi, Y., Kochian, L.V. and Wu, R.J. (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proc. Natl Acad. Sci. USA*, **99**, 15898–15903.

Gavrieli, Y., Sherman, Y. and Ben-Sasson, S.A. (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493–501.

Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D. and Thomashow, M.F. (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol.* **124**, 1854–1865.

Ha, H.C. and Snyder, S.H. (1999) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. *Proc. Natl Acad. Sci. USA*, **96**, 13978–13982.

Helliwell, C. and Waterhouse, P. (2003) Constructs and methods for high-throughput gene silencing in plants. *Methods*, **30**, 289–295.

Hoeberichts, F.A. and Woltering, E.J. (2003) Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. *BioEssays*, **25**, 47–57.

Holzberg, S., Brosio, P., Gross, C. and Pogue, G.P. (2002) Barley stripe mosaic virus-induced gene silencing in a monocot plant. *Plant J.* **30**, 315–327.

Jang, I.-C., Oh, S.-J., Seo, J.-S. *et al.* (2003) Expression of a bifunctional fusion of the Escherichia coli genes for threhalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. *Plant Physiol.* **131**, 516–524.

Kandasamy, M.K., McKinney, E.C. and Meagher, R.B. (2002) Functional nonequivalency of actin isovariants in *Arabidopsis*. *Mol. Biol. Cell*, **13**, 251–261.

Karp, M.T., Raunio, R.P. and Lövgren, T.N.-E. (1983) Simultaneous extraction and combined bioluminescent assay of NAD+ and NADH. *Anal. Biochem.* **128**, 175–180.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol.* **17**, 287–291.

Kawai-Yamada, M., Jin, L., Yoshinaga, K., Hirata, A. and Uchimiya, H. (2001) Mammalian Bax-induced plant cell death can be down-regulated by overexpression of Arabidopsis Bax Inhibitor-1 (AtBI-1). *Proc. Natl Acad. Sci. USA*, **98**, 12295–12300.

Klaidman, L.K., Yang, J., Chang, M.L. and Adams, J.D., Jr (2003) Recent developments on the role of mitochondria in poly(ADP-ribose) polymerase inhibition. *Curr. Med. Chem.* **10**, 2669–2678.

Knight, H. and Knight, M.R. (2001) Abiotic stress signalling pathways: specificity and cross-talk. *Trends Plant Sci.* **6**, 262–267.

Lincoln, J.E., Richael, C., Overduin, B., Smith, K., Bostock, R. and Gilchrist, D.G. (2002) Expression of the antiapoptotic baculovirus p35 gene in tomato blocks programmed cell death and provides broad-spectrum resistance to disease. *Proc. Natl Acad. Sci. USA*, **99**, 15127–15221.

Matsumura, H., Nirasawa, S., Kiba, A., Urasaki, N., Saitoh, H., Ito, M., Kawai-Yamada, M., Uchimiya, H. and Terauchi, R. (2003) Overexpression of Bax inhibitor suppresses the fungal elicitor-induced cell death in rice (*Oryza sativa* L) cells. *Plant J.* **33**, 425–434.

Ménissier de Murcia, J., Ricoul, M., Tartier, L. *et al.* (2003) Functional interaction between PARP-1 and PARP-2 in chromosome stability and embryonic development in mouse. *EMBO J.* **22**, 2255–2263.

Meskiene, I. and Hirt, H. (2000) MAP kinase pathways: molecular plug-and-play chips for the cell. *Plant Mol. Biol.* **42**, 791–806.

Moon, H., Lee, B., Choi, G. *et al.* (2003a) NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. *Proc. Natl Acad. Sci. USA*, **100**, 358–363.

Moon, H.J., Baek, D.W., Lee, J.Y., Nam, J.S. and Yun, D.-J. (2003b) Functional screening for cell death suppressors and development of multiple stress-tolerant plants. *J. Plant Biotechnol.* **5**, 143–148.

Murrell, T.S. and Childs, F.R. (2000) Redefining corn yield potential. *Better Crops*, **84**, 33–37.

Nakamura, J., Asakura, S., Hester, S.D., de Murcia, G., Caldecott, K.W. and Swenberg, J.A. (2003) Quantitation of intracellular NAD(P)H can monitor an imbalance of DNA single strand break repair in base excision repair deficient cells in real time. *Nucleic Acids Res.* **31**, e104.

Odell, J.T., Nagy, F. and Chua, N.H. (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature*, **313**, 810–812.

del Pozo, O. and Lam, E. (2003) Expression of the baculovirus p35 protein in tobacco affects cell death progression and compromises N gene-mediated disease resistance response to Tobacco mosaic virus. *Mol. Plant Microbe Interact.* **16**, 485–494.

Puchta, H., Swoboda, P. and Hohn, B. (1995) Induction of intrachromosomal homologous recombination in whole plants. *Plant J.* **7**, 203–210.

Qiao, J., Mitsuhara, I., Yazaki, Y., Sakano, K., Gotoh, Y., Miura, M. and Ohashi, Y. (2002) Enhanced resistance to salt, cold and wound stresses by overproduction of animal cell death suppressors Bcl-xL and Ced-9 in tobacco cells – their possible contribution through improved function in organella. *Plant Cell Physiol.* **43**, 992–1005.

Rawyler, A., Pavelic, D., Gianinazzi, C., Oberson, J. and Braendle, R. (1999) Membrane lipid integrity relies on a threshold of ATP production rate in potato cell cultures submitted to anoxia. *Plant Physiol.* **120**, 293–300.

Ricci, J.-E., Waterhouse, N. and Green, D.R. (2003) Mitochondrial functions during cell death, a complex (I-V) dilemma. *Cell Death Differ.* **10**, 488–492.

Rizhsky, L., Liang, H. and Mittler, R. (2002) The combined effect of drought stress and heat shock on gene expression in tobacco. *Plant Physiol.* **130**, 1143–1151.

Sanfacon, H., Brodmann, P. and Hohn, T. (1991) A dissection of the cauliflower mosaic virus polyadenylation signal. *Genes Dev.* **5**, 141–149.

Schreiber, V., Amé, J.-C., Dollé, P., Schultz, I., Rinaldi, B., Fraulob, V., Ménissier-de Murcia, J. and de Murcia, G. (2002) Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1. *J. Biol. Chem.* **277**, 23028–23036.

Shou, H., Bordallo, P., Fan, J.-B., Yeakley, J.M., Bibikova, M., Sheen, J. and Wang, K. (2004) Expression of an active tobacco mitogen-activated protein kinase kinase kinase enhances freezing tolerance in transgenic maize. *Proc. Natl Acad. Sci. USA*, **101**, 3298–3303.

Southan, G.J. and Szabó, C. (2003) Poly(ADP-ribose) polymerase inhibitors. *Curr. Med. Chem.* **10**, 321–340.

Tiwari, B.S., Belenghi, B. and Levine, A. (2002) Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. *Proc. Natl Acad. Sci. USA*, **128**, 1271–1281.

Tollenaar, M. and Lee, E.A. (2002) Yield potential, yield stability and stress tolerance in maize. *Field Crop Res.* **75**, 161–169.

Tollenaar, M. and Wu, J. (1999) Yield improvement in temperature maize is attributable to greater stress tolerance. *Crop Sci.* **39**, 1597–1604.

Trucco, C., Oliver, F.J., de Murcia, G. and Ménissier-de Murcia, J. (1998) DNA repair defect in poly(ADP-ribose) polymerase-deficient cell lines. *Nucleic Acids Res.* **26**, 2644–2649.

Virág, L. and Szabó, C. (2002) The therapeutic potential of poly(-ADP-ribose) polymerase inhibitors. *Pharmacol. Rev.* **54**, 375–429.

Wang, W., Vinocur, B. and Altman, A. (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*, **218**, 1–14.

Waterhouse, P.M., Graham, M.W. and Wang, M.-B. (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl Acad. Sci. USA*, **95**, 13959–13964.

Waterhouse, P.M., Wang, M.-B. and Finnegan, E.J. (2001) Role of short RNAs in gene silencing. *Trends Plant Sci.* **6**, 297–301.

Xiong, L., Schumaker, K.S. and Zhu, J.-K. (2002) Cell signaling during cold, drought, and salt stress. *Plant Cell*, **??**(Suppl), S165–S183.

Zhu, J.-K. (2001) Cell signaling under salt, water and cold stresses. *Curr. Opin. Plant Biol.* **4**, 401–406.

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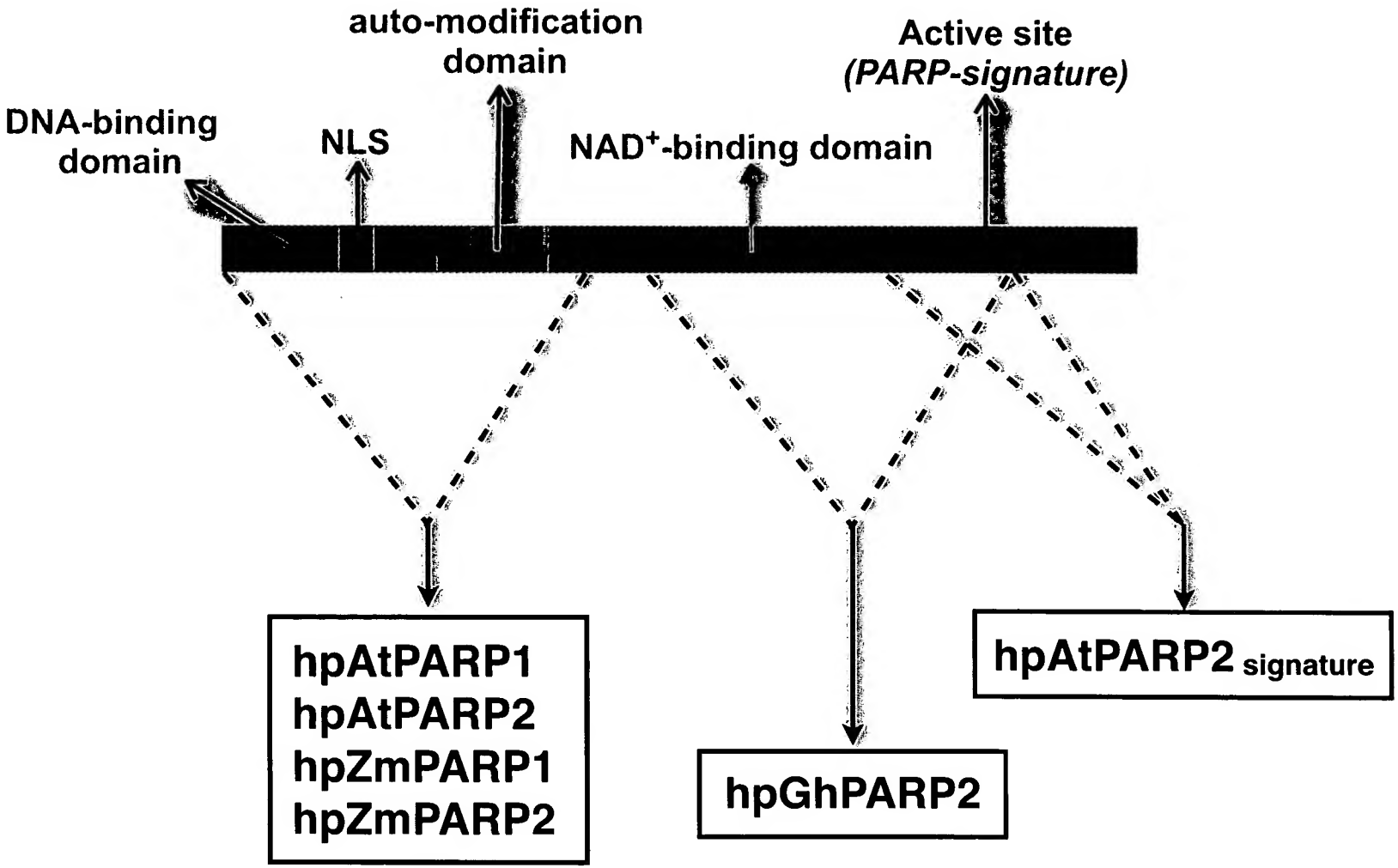
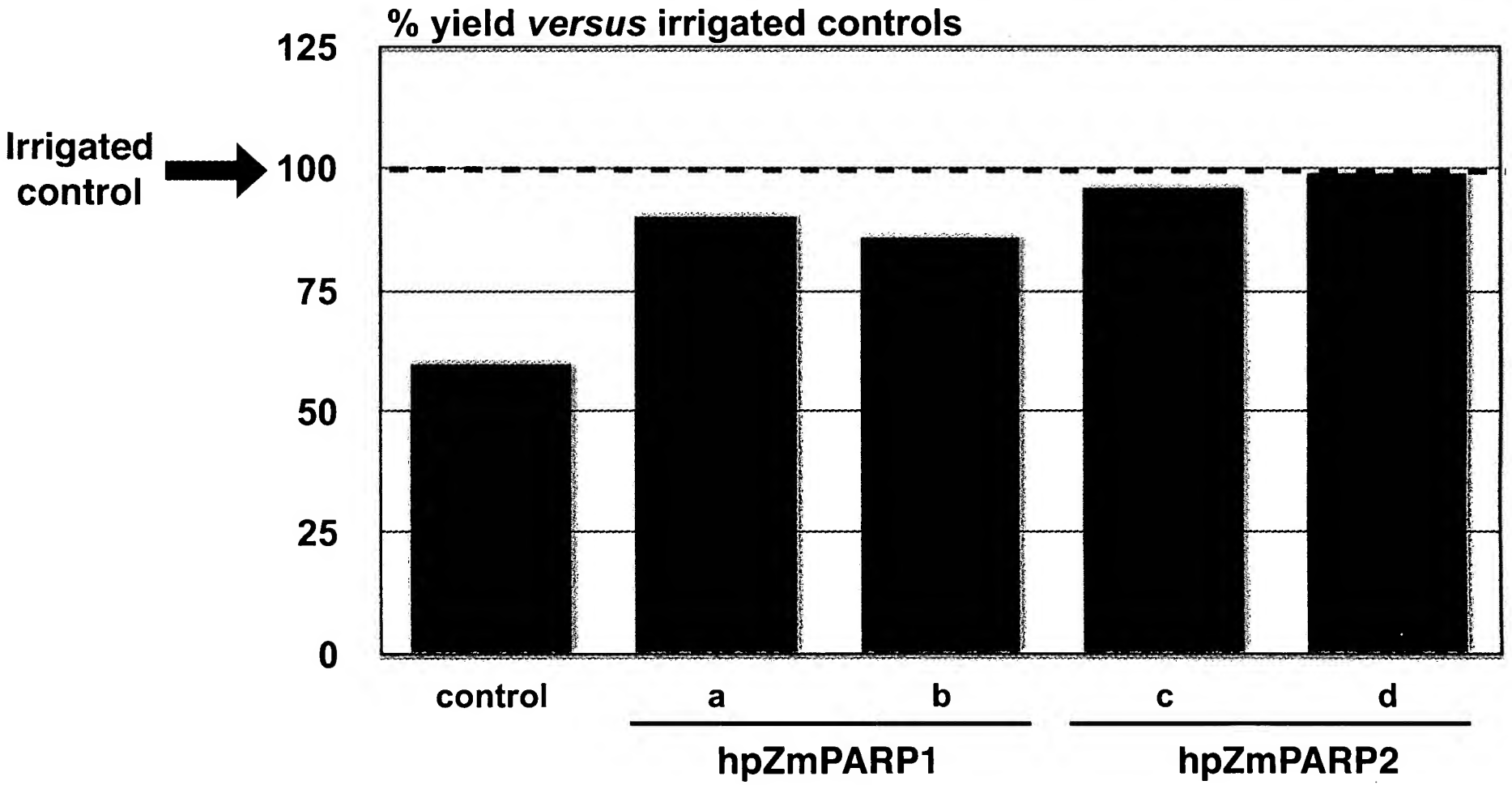
Improving stress tolerance and yield
A genetic and an epigenetic approach

De Block
Second Supplemental Declaration
ANNEX II

Silencing of the *PARP*-genes



hpPARP-corn lines: Drought stress tolerance in the field



1-10 % PAR-activity *versus* control under stress condition